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Title: Use of Hydrogen Peroxide in Combination with Nisin, Sodium Lactate and Citric Acid for Reducing Transfer of Bacterial Pathogens from Whole Melon Surfaces to Fresh-Cut Pieces

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Use of hydrogen peroxide in combination with nisin, sodium lactate and citric acid for reducing transfer of bacterial pathogens from whole melon surfaces to fresh-cut pieces☆

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Abstract

Hydrogen peroxide (2.5%) alone or hydrogen peroxide (1%) in combination with nisin (25 μg/ml), sodium lactate (1%), and citric acid (0.5%) (HPLNC) were investigated as potential sanitizers for reducing *Escherichia coli* O157:H7 or *Listeria monocytogenes* populations on whole cantaloupe and honeydew melons. Whole cantaloupes inoculated with *E. coli* O157:H7 and *L. monocytogenes* at 5.27 and 4.07 $\log_{10}$ CFU/cm^2, respectively, and whole honeydew melons inoculated with *E. coli* O157:H7 and *L. monocytogenes* at 3.45 and 3.05 $\log_{10}$ CFU/cm^2, respectively, were stored at 5 °C for 7 days. Antimicrobial washing treatments were applied to inoculated whole melons on days 0 or 7 of storage and surviving bacterial populations and the numbers transferred to fresh-cut pieces were determined. At days 0 and 7 treatment with HPLNC significantly ($p<0.05$) reduced the numbers of both pathogens, by 3 to 4 log CFU/cm^2 on both types of whole melon. Treatment with HPLNC was significantly ($p<0.05$) more effective than treatment with 2.5% hydrogen peroxide. While fresh-cut pieces prepared from stored whole melons were negative for the pathogens by both direct plating and by enrichment, fresh-cut pieces from cantaloupe melons treated with 2.5% hydrogen peroxide were positive for both pathogens and pieces from honeydew melons were positive for *E. coli* 0157:H7. The native microflora on fresh-cut melons were also substantially reduced by HPLNC treatment of whole melons. The results suggest that HPLNC could be used to decontaminate whole melon surfaces and so improve the microbial safety and quality of fresh-cut melons.

Keywords: Cantaloupe; Honeydew; Fresh-cut pieces; *Listeria monocytogenes*; *E. coli*; HPLNC; H_2O_2

☆ Mention of trade names or commercial products is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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1. Introduction

Cantaloupe (*Cucumis melo* L. var. *cantalupenis* Naud.) and honeydew (*Cucumis melo* L. var. *inodorus* Naud.) melons are popular fruits in the United States and other parts of the world. The surfaces of cantaloupes are covered with a meshwork of raised tissue commonly referred to as the "net" (Webster and Craig, 1976) while honeydews lacks the "net" tissue. Melon surfaces, like most vegetables, are frequently in contact with the soil (Brackett, 1992; Beuchat, 1995) and their surfaces will carry contaminants which are easily transferred to the melon pieces during preparation of fresh-cut (Ukuku and Sapers, 2001; Ukuku and Fett, 2002a). There are many reports of disease due to consumption of fruits and vegetables that were contaminated on the surface with enteric pathogens (Beuchat, 1995). Therefore, the safety of fresh-cut melons and other produce available in salad-bar operations and supermarkets is a concern (Hurst and Schuler, 1992; FDA, 2001).

Enterohemorrhagic *Escherichia coli* O157:H7 is recognized as an important foodborne pathogen (Doyle, 1991; Madden, 1992; Padhye and Doyle, 1992). In August, 1993, an outbreak of foodborne illness was linked to eating cantaloupe contaminated with *E. coli* O157:H7 (M. Diermayer, Oregon Health Division, Portland, OR, personal communication). Although there are no documented reports of outbreaks of human listeriosis associated with the consumption of fresh-cut cantaloupe or honeydew melons, the potential for such an outbreak remains a concern as evidenced by a recent recall of fresh-cut melons (FDA, 2003) and fresh-cut fruit salad due to possible contamination with *Listeria monocytogenes* (Kroger, 2000). Minimally processed fresh-cut fruits and vegetables provide a good substrate for microbial growth (Marston, 1995; Nguyen-the and Carlin, 1994). Such substrate may allow proliferation of human pathogenic organisms like enterohemorrhagic *E. coli* and *L. monocytogenes*.

Washing is one of the first processing operations to which a fruit or vegetable is subjected. Wash water chlorinated up to 200 ppm is routinely applied to reduce microbial contamination in produce processing lines (Wei et al., 1985). However, the use of chlorine is of concern due to the potential formation of harmful by-products (Richardson et al., 1998) and can only achieve approximately 2 to 3 log reductions of native microflora (Ukuku et al., 2001). Thus, there is much interest in developing safer and more effective sanitizers for fruits and vegetables. Ukuku and Fett (2002a) reported that nisin in combination with EDTA reduced microbial spoilage of whole and fresh-cut melons and extended the shelf. In another study, Ukuku and Fett (2004) reported reduction of *Salmonella* populations on whole and fresh-cut cantaloupe treated with nisin in combination with sodium lactate and potassium sorbate. The use of hydrogen peroxide (2.5% to 5.0%) washes for reducing native microflora and *Salmonella* populations on inoculated whole and minimally processed fresh-cut cantaloupe has been investigated also (Ukuku et al., 2001; Ukuku, 2004). Total inactivation of inoculated *Salmonella* populations was not achieved (Ukuku, 2004). The purpose of the present study was to investigate the efficacy of hydrogen peroxide treatments in combination with nisin, sodium lactate and citric acid as a mixed antimicrobial wash solution for inactivating *E. coli* O157:H7 and *L. monocytogenes* populations inoculated on whole cantaloupe and honeydew melon surfaces. Also, the effect of the antimicrobial mixture in reducing the transfer of inoculated human bacterial pathogens from the treated whole cantaloupe and honeydew melon surfaces to fresh-cut pieces was investigated.

2. Materials and methods

2.1. Bacterial strains, growth conditions, and preparation

Bacteria used in this study were *E. coli* O157:H7 strains SEA13B88 and Oklahoma which were indicted in apple cider-related outbreaks; and *L. monocytogenes* strains Scott A, a clinical isolate, and CCR1-L-G, a food isolate. Bacterial strains were obtained from the USDA-ARS-ERRC culture collection. Bacteria were maintained on Brain Heart Infusion Agar (BHIA; BBL/Difco, Sparks, MD, USA) slants held at 4 °C. Prior to use, the cultures were subjected to two successive transfers by loop inocula to 5 ml Brain Heart Infusion Broth (BHIB; BBL/Difco) for *E. coli*, or 5 ml Trypticase Soy Broth supplemented with 0.6% yeast extract (TSBYE, BBL/Difco) for *L. monocytogenes*. A final transfer of 0.2 ml was made into 20 ml BHIB or TSBYE with incubation at 36 °C for 18 h under static conditions. The bacteria were harvested by centrifuga-

tion (10,000×*g*, 10 min) at 4 °C. The cell pellets were washed in a salt–peptone solution [0.85% NaCl, 0.05% Bacto–peptone (BBL/Difco)]. The cell pellets were used to prepare two different types of inocula. One type of inoculum consisted of a mixture of the two strains of *E. coli* O157:H7 each at numbers ranging from 1.7 to 2.2×10^7 CFU/ml. The second inoculum type consisted of a mixture of the two strains of *L. monocytogenes* each at numbers ranging from 2.2 to 2.5×10^7 CFU/ml.

2.2. Inoculation of whole melon

Unwaxed whole "Western shippers" cantaloupes (1665 g to 1718 g) and honeydew melons (1698 g to 1763 g) purchased from a local distributor were placed on a bench top for 18–20 h to allowed the melons to come to room temperature (~20 °C) before being inoculated. Both cantaloupes and honeydew melons were submerged in 3 l of bacterial inoculum and were rotated with a glove-covered hand for 10 min to ensure even inoculation. After inoculation, the cantaloupes were placed inside a biosafety cabinet to dry for 1 h and then were stored at 5 °C for up to 7 days before washing with antimicrobial agents.

2.3. Preparation of antimicrobial solutions for washing treatments

A stock solution of nisin (10^6 I. U.; Sigma, St. Louis, MO, USA) was prepared at a concentration of 2500 μg/ml in 0.02 N HCl. Sodium lactate (NaL, 60% w/v; Sigma) was prepared at a concentration of 20% in deionized distilled water (ddH_2O). Citric acid (CA; Aldrich, Milwaukee, WI, USA) was prepared at a concentration of 10% in ddH_2O. These stock solutions were sterilized by filtration through 0.22 μm filters (Millipore, Bedford, MA, USA). Hydrogen peroxide was prepared from a 30% stock solution (Fisher Scientific, Suwanee, GA, USA) by dilution with sterile tap water.

2.4. Washing treatment

Melons were washed with sterile tap water, 2.5% H_2O_2, 25 μg/ml nisin, 1% NaL or 0.5% CA, or a solution of 1% H_2O_2, 25 μg/ml nisin, 1% NaL and 0.5% CA (HPLNC). All solutions were prepared in sterile tap water at a temperature of about 19 °C. The pH of the wash solutions was adjusted to 6.7 ± 0.1 by adding 2 N sodium hydroxide (NaOH), a pH that approximated the pH of cantaloupe flesh. All washing treatments, on 0 and 7 days post inoculation, were performed by submerging the melons under the surface of 3 l of wash solution, and rotating by hand for 5 min to assure complete coverage of surfaces with solution. Washed melons were allowed to dry for 1 h in a biosafety cabinet before cutting.

2.5. Microbiological analyses

A sterilized stainless steel cork-borer was used to cut through whole melon surfaces at random locations to produce rind plugs 22 mm in diameter with a rind surface area of 3.80 cm^2. Flesh adhering to the rind plugs was trimmed off using a sterilized stainless steel knife. Forty-five melon rind plugs per whole melon, weighing approximately 20 g in total, were blended using a Waring commercial blender (Dynamic Corp., New Hartford, CT, USA) with the speed set at level 5, for 1 min, with 80 ml of sterile 0.1% peptone water. Decimal dilutions of the sample were made with 0.1% peptone water (BBL/Difco), and 0.1 ml portions were plated in duplicate on Plate Count Agar (PCA; BBL/Difco) which were incubated at 30 °C for 72 h, for enumeration of mesophilic aerobic bacteria; and plates of Potato Dextrose Agar (BBL/Difco) acidified with 10% tartaric acid (PDAA) to pH 3.5 which were incubated at 25 °C for 5 days, for enumeration of yeast and molds.

For *L. monocytogenes*, *Listeria* identification agar (PALCAM; Sigma) containing *Listeria* selective supplement (L-4660; Sigma) was used, with incubation of plates at 37 °C for 48 h. All plating was done in duplicate. In addition, pure cultures of *L. monocytogenes* were surface plated onto PALCAM agar to serve as a reference for identification. Representative, presumptive colonies of *L. monocytogenes* were confirmed using API *Listeria* test kits (bioMeriux, Marcy l'Etiole, France). For enumeration of *E. coli* 0157:H7, plating was done on Sorbitol MacConkey Agar containing potassium tellurite and cefixime (TC-SMAC; BBL/Difco) with incubation of plates at 37 °C for 24 h. Selected colonies were confirmed to be *E. coli* as described by Hitchins et al. (1995).

2.6. Fresh-cut melons

To prepare fresh-cut pieces, whole melons were cut into four sections using a sterile knife and the rinds were carefully removed. The interior flesh was cut into ~3 cm cubes and fresh-cut pieces were investigated for the presence of transferred *E. coli* O157:H7 or *L. monocytogenes*. Approximately 100 g of the flesh were placed in a Stomacher® bag with 200 ml of 0.1% peptone water and pummeled for 30 s in a Stomacher (model 400; Dynatech Laboratories, Alexandria, VA, USA) at medium speed. Samples (1 ml) were pour plated using either TC-SMAC or PALCAM containing *Listeria* selective supplement and plates were incubated at 37 °C for 48 h (Lovett and Hitchins, 1988). In a separate experiment, fresh-cut pieces (~100 g) were placed in a Stomacher® bag with 200 ml of University of Vermont (UVM) broth (BBL/Difco) followed by incubation at 35 °C for 24 h. A 1 ml portion of the UVM broth culture was added to 9 ml of Fraser broth (BBL/Difco) and incubated at 35 °C for 24 h. An A.O.A.C. approved *Listeria* Rapid Test (Oxoid, Ogdensburg, NY, USA) was used to test for the presence of *L. monocytogenes* in the broth culture. For *E. coli* O157:H7, 200 ml of tryptic soya broth (TSB; BBL/Difco) was added to the same amount of fresh-cut melon in a stomacher bag. After incubation at 37 °C for 6 h, 0.1 ml portions of the homogenates was plated on TC-SMAC and plates were incubated at 37 °C for 24 h. Selected colonies were confirmed as *E. coli* as described by Hitchins et al. (1995).

2.7. Data analysis

The responses of *L. monocytogenes* and *E. coli* 0157:H7 to sanitizer treatments were analyzed by performing analysis of variance to determine the effect of treatments. Differences between treatments

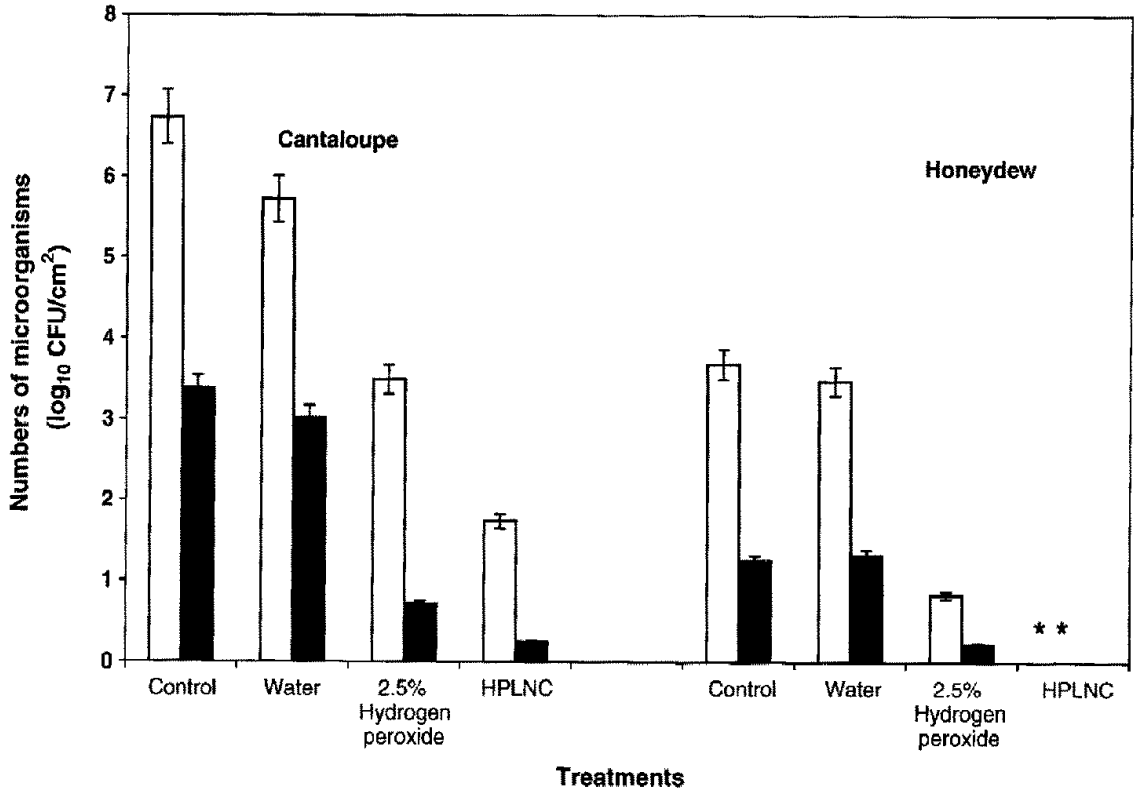

Fig. 1. Populations of mesophilic aerobes (□) and yeasts and molds (■) on the surface of treated and untreated whole cantaloupe and honeydew melons. Values represents mean ± S.E. of values from three separate experiments. *<20 CFU/cm^2; HPLNC, washing solution containing H_2O_2 (1%)+nisin (25 μg/ml)+NaL (1%)+CA (0.5%).

were performed using the Bonferroni's least significant difference (LSD) mean separation procedure (Miller, 1981) at the $p=0.05$ significance level using the 1989 SAS program (SAS Institute, Cary, NC., USA).

3. Results and discussion

3.1. Effect of washing treatments on aerobic microflora of whole and fresh-cut melons

In commercial operations, melons are often treated with antimicrobials and/or wax to retard invasion by spoilage organisms (O'Connor-Shaw et al., 1994). However, some growers are now packing melons in the field for direct shipment, and treatments intended to retard spoilage are not applied. In preliminary experiments, washing whole melons with water, H_2O_2, NaL, CA, or nisin alone did not cause significant ($p>0.05$) reductions in aerobic mesophilic bacteria on the surfaces of whole melons. The use of 2.5% H_2O_2 caused significant ($p<0.05$) reductions in populations of aerobic mesophilic bacteria and yeast and mold on cantaloupe and honeydew melon surfaces (Fig. 1). However, treatment of whole melon surfaces with HPLNC was significantly ($p<0.05$) more effective than washing with water or 2.5% H_2O_2. Populations of aerobic mesophilic bacteria and yeast and mold on honeydew melon surfaces treated with HPLNC were below the detection levels of <20 CFU/cm^2 for aerobic mesophiles and <1 CFU for yeast and mold.

Populations of aerobic mesophilic bacteria and yeast and mold transferred during cutting, to fresh-cut pieces from unwashed or water washed whole melons, and from melons treated with 2.5% H_2O_2 or HPLNC are shown in Fig. 2. Populations of aerobic mesophilic bacteria and yeasts and molds in pieces cut from whole melons treated with H_2O_2 and HPLNC were significantly ($p<0.05$) lower than fresh-cut pieces from unwashed and water washed melons.

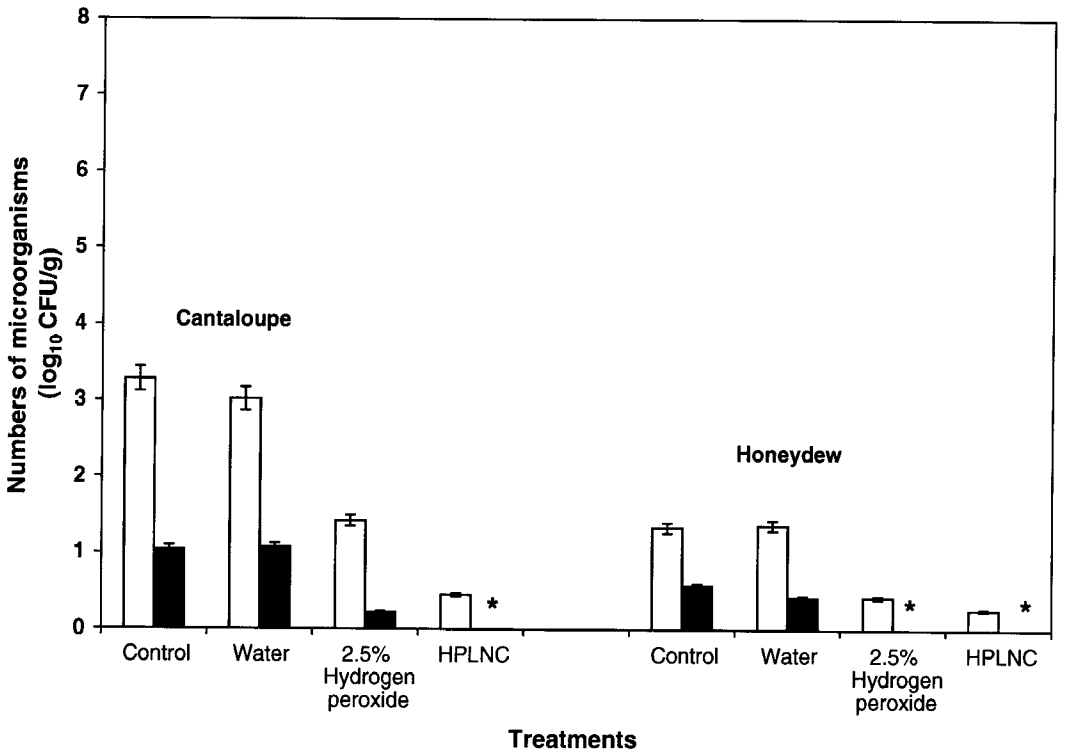

Fig. 2. Recovery of mesophilic aerobes (□) and yeasts and molds (■) in fresh-cut melons prepared from treated and untreated whole cantaloupe and honeydew melons. Values represent mean ± S.E. of values from three separate experiments. *<1 CFU/g.

Yeasts and molds were not detected in fresh-cut pieces prepared from HPLNC treated cantaloupe and honeydew melons.

3.2. Effect of washing treatments on L. monocytogenes and E. coli O157:H7 inoculated onto whole melons

Populations of *L. monocytogenes* were significantly ($p>0.05$) higher on cantaloupe than on honeydew surfaces (Fig. 3). These populations did not significantly ($p>0.05$) change during storage at 5 °C for up to 7 days. In preliminary experiments, washing inoculated whole melons at day 0 with water did not cause significant ($p>0.05$) reductions in *L. monocytogenes* populations. However, NaL (1%), CA (0.5%), and nisin (25 μg/ml) individually caused approximately 0.8 log reductions of *L. monocytogenes* (data not shown). Treatment with 2.5% H_2O_2 resulted in approximately 3 log reductions of *L. monocytogenes* on both types of melon while no *L. monocytogenes* were detected on melons treated with HPLNC.

Populations of *E. coli* O157:H7 were significantly ($p<0.05$) higher on cantaloupes than on honeydew surfaces (Fig. 4). The higher populations of *E. coli* O157:H7 and *L. monocytogenes* recovered from the surfaces of cantaloupe was likely due to the ruptured meshwork of raised tissue (the net) on the cantaloupe. This net consists of lenticels and phellum (cork) cells which presents a variety of surfaces to which bacteria may bind (Ukuku and Fett, 2002a,b).

Washing inoculated melons at day 0 with water, NaL, CA or nisin, did not cause significant ($p>0.05$) reductions in *E. coli* O157:H7 populations (data not shown). HPLNC reduced *E. coli* O157:H7 numbers on cantaloupe surfaces by 4 log units and no *E. coli* O157:H7 were recovered from treated honeydew melon surfaces. Treatment of inoculated cantaloupe and honeydew melons with 2.5% hydrogen peroxide

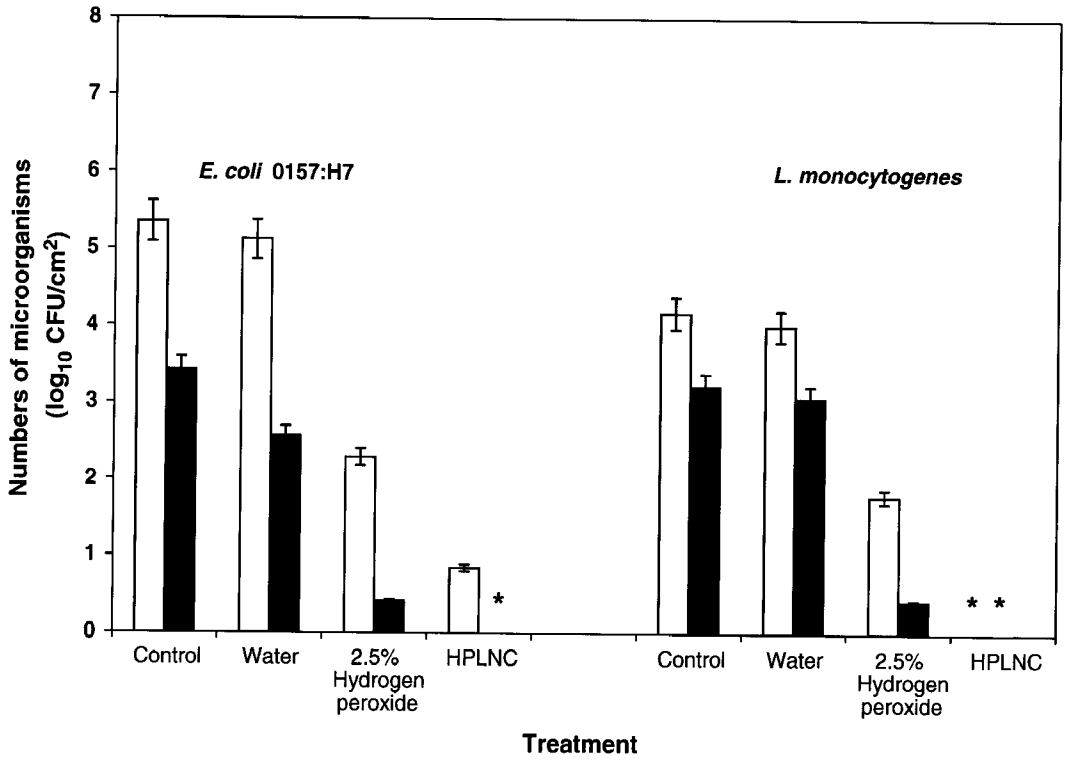

Fig. 3. Effect of HPLNC and 2.5% H_2O_2 treatment on the survival of *L. monocytogenes* or *E. coli* O157:H7 populations on whole cantaloupe (□) or honeydew (■) melon at day 0 of contamination and treatment. Values represent mean ± S.E. of values from three separate experiments. *<20 CFU/cm^2; HPLNC, washing solution containing H_2O_2 (1%)+nisin (25 μg/ml)+NaL (1%)+CA (0.5%).

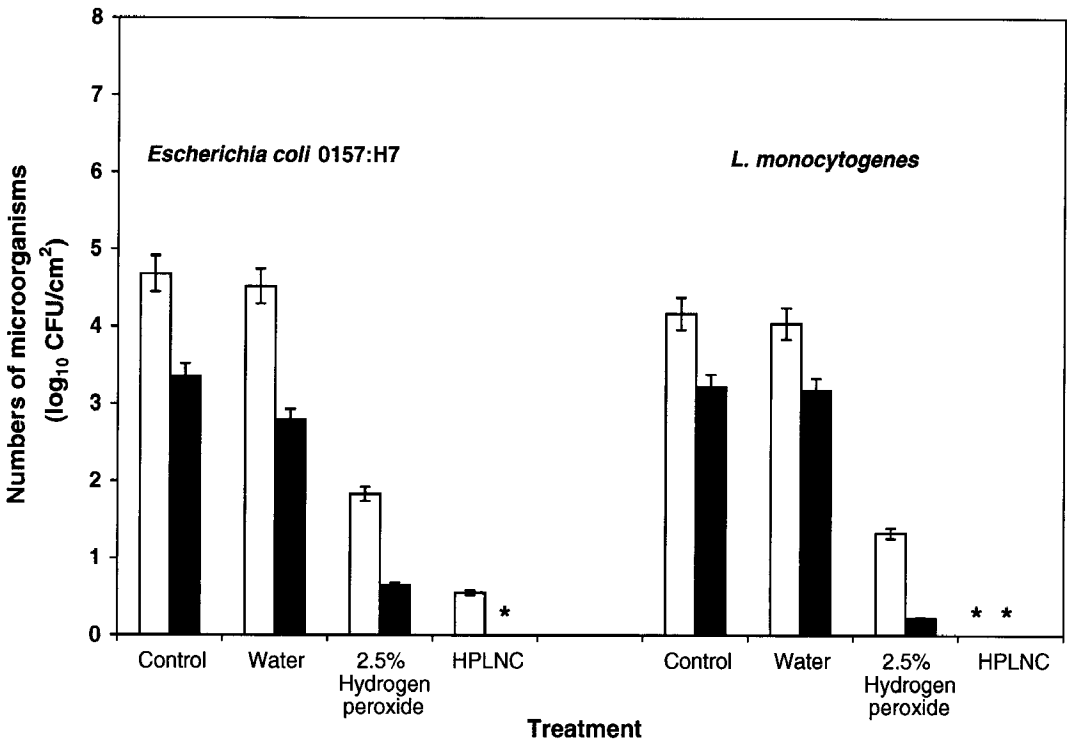

Fig. 4. Effect of HPLNC and 2.5% H_2O_2 treatment on the survival of *L. monocytogenes* or *E. coli* O157:H7 on whole cantaloupe (□) or honeydew (■) melon stored at 5 °C for 7 days after inoculation. Values represent mean ± S.E. of values from three separate experiments. *<20 CFU/cm^2; HPLNC, washing solution containing H_2O_2 (1%)+nisin (25 μg/ml)+NaL (1%)+CA (0.5%).

resulted in approximate 3 log reductions of *E. coli* O157:H7 populations on both types of melons.

Previously, 2 to 3 log reductions of *E. coli* ATCC 25922 (Ukuku et al., 2001) or *Salmonella* (Ukuku, 2004) inoculated on whole cantaloupe by washing with 5% H_2O_2 were reported. Also, bacteria inoculated on the surfaces of melons that were stored for more than 2 days before applying sanitizer treatments were difficult to remove. The strength of attachment to the melon surface was indicated by S_R-values for retention of the pathogen on melon surfaces that underwent washing (Ukuku and Fett, 2002a,b).

Populations of *E. coli* O157:H7 on melon surfaces slightly decreased while *L. monocytogenes* remain the same during storage at 5 °C for 7 days (Figs. 3 and 4). When whole melons inoculated with *E. coli* O157:H7 or *L. monocytogenes* were stored at 5 °C and treated with HPLNC after day 7 the reductions of both pathogens were significantly ($p<0.05$) greater than on the melons treated with H_2O_2 (2.5%) alone (Fig. 4).

In this study, treatments with nisin, NaL or CA alone did not cause significant inactivation of *E. coli* O157:H7 on melon surfaces, but populations of *L. monocytogenes* on both types of melons were slightly reduced. In a previous study, treatment of cantaloupe melons with water, EDTA (0.02 M) or nisin (10 μg/ml) alone did not cause significant reductions of native microflora on whole cantaloupe (Ukuku and Fett, 2002a). However, when tested in combination, at day 0, the numbers of bacteria on cantaloupe surfaces were reduced by approximately 3 log units (Ukuku and Fett, 2004). The reductions for *E. coli* O157:H7 using HPLNC were better than those previously reported for treatments with chlorine (1000 ppm) or H_2O_2 (2.5% to 5%) (Ukuku et al., 2001).

Significant reductions of *Escherichia coli* O157:H7 and *Salmonella* populations on animal carcass surfaces sprayed with lactic acid (2%) were reported by Castillo et al. (2001). Also, sodium, potassium and calcium lactates (2–4%) have been shown

Table 1
Recovery of pathogens from the rinds of groups of 6 melons after inoculation and storage of melons at 5 °C for 0 or 7 days and treatment of whole melons to reduce transfer of pathogens from melon rind to fresh-cut pieces

Treatment	Melons yielding positive pieces							
	Cantaloupe				Honeydew			
	E. coli O157:H7		*L. monocytogenes*		*E. coli* O157:H7		*L. monocytogenes*	
	Day 0	Day 7	Day 0	Day 7	Day 0	Day 7	Day 0	Day 7
Control	6	6	6	6	6	6	6	6
Water	6	6	6	6	6	6	6	6
2.5% H_2O_2	1 (2)	3 (3)	0 (1)	1 (3)	0 (0)	2 (2)	0 (0)	0 (0)
HPLNC[a]	0 (0)[b]	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

[a] HPLNC=a mixture of H_2O_2 (1%)+nisin (25 μg/ml)+NaL (1%)+CA (0.5%).
[b] Numbers in parentheses represent fresh-cut pieces that were negative by direct plating but positive after enrichment.

to control growth of aerobes and anaerobes in meats, and to have antibotulinal and antilisterial activities (Mass et al., 1989; Papadopoulos et al., 1991; Shelef, 1994; Shelef and Yang, 1991). Chemical or physical antimicrobial treatments of whole melons before shipment and fresh-cut preparation may be desirable. However, the recontamination of whole melons with *L. monocytogenes* or *E. coli* 0157:H7 after sanitizing whole melon surfaces, because of poor plant sanitation or use of improper handling or packaging procedures, would then still be a food safety concern (Ukuku and Fett, 2002b).

3.3. Transfer of E. coli 0157:H7 or L. monocytogenes from the melon surface to fresh-cut cubes

At the retail level or at food establishments, melons that are to be cut are usually washed using only potable water. Thus, fresh-cut melon may not be adequately protected from contamination. Results of experiments designed to assess the transfer of *E. coli* or *L. monocytogenes* on melon rind to fresh-cut pieces are shown in Table 1. Populations of *E. coli* or *L. monocytogenes* on fresh-cut pieces prepared from untreated whole melons stored at 5 °C for 0 and 7 days were not significantly ($p>0.05$) different from population on pieces from water washed melons. Populations of *E. coli* on fresh-cut pieces prepared from 2.5% H_2O_2 treated whole melons stored at 5 °C for 0 and 7 days were below the detection levels by direct plating. Enrichments of fresh-cut pieces from 2.5% H_2O_2 treated melons that were negative at day 0 by direct plating were positive for survivors of *E. coli* O157:H7 but were negative for *L. monocytogenes* (Table 1). Fresh-cut pieces from 2.5% H_2O_2 treated whole melon that were negative at day 7 by direct plating were positive for *E. coli* O157:H7 and *L. monocytogenes* by enrichment except for fresh-cut honeydew from which no *L. monocytogenes* was recovered. Populations of *E. coli* or *L. monocytogenes* on fresh-cut pieces prepared from HPLNC treated whole melons at day 0 were below the detection levels by both direct plating and enrichment. Enrichments of fresh-cut melons at day 0 prepared from HPLNC treated whole melons were negative for *E. coli* O157:H7 and *L. monocytogenes*. Fresh-cut pieces from melons treated with HPLNC at day 7 that were negative for *E. coli* O157:H7 and *L. monocytogenes* by direct plating were also negative by enrichment.

The efficacy of hydrogen peroxide alone for preservation of fresh-cut melon (Sapers et al., 2001), for reducing populations of *Salmonella* inoculated on cantaloupe surfaces (Ukuku, 2004), and for washing of fresh mushrooms (Sapers et al., 1994) has been reported. Previously, it was reported that treatment of fresh-cut melons with nisin in combination with NaL improved the microbial quality by delaying growth of spoilage microflora (Ukuku and Fett, 2004). In this study, HPLNC gave a better results than other treatments.

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